Acta med. Nagasaki. 12: 18–29

Amino Acid Sequences of Tryptic Peptides, $^{\alpha}$T9, $^{\alpha}$T10, and $^{\alpha}$T11 from the $^{\alpha}$-Polypeptide Chain in *Macaca mulatta* Monkey Hemoglobin

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Received for publication, June 5,1968

A partial amino acid sequence of the α-polypeptide chain in hemoglobin from *macaca mulatta* monkey was determined and compared with the known sequence of the corresponding part of human hemoglobin. Two differences were discovered between them.

The α-polypeptide chain of *macaca mulatta* monkey hemoglobin was hydrolyzed with trypsin. Three tryptic peptides, αT9, αT10, and αT11 were the subjects of this experiment. αT9 was further hydrolyzed with pepsin and then the amino acid sequence of αT9 was determined by arranging partial sequences of those peptic peptides which were determined by PTC and DNP methods. The sequences of αT10 and αT11 were directly determined by PTC and DNP methods. The comparison of the results thus obtained with the known sequence of human hemoglobin showed that there were two differences only in αT9. Namely, the seventh and the tenth amino acid residues from tne N-terminus were respectively asparagine and alanine in human hemoglobin, but leucine and glycine in *macaca mulatta* monkey hemoglobin.

INTRODUCTION

Problems on the evolution of living things have in general been discussed from the viewpoint of comparative studies of their physical shapes and their habits. Considering that they are all of chemical constituents, however, the comparative studies of their chemical components on molecular level can also be very suggestive to this kind of problems. By the comparison of the structures of proteins from various kinds of living things, ANFINSEN[1] presumed the evolutional process of gene which is considered to be the regulator of protein biosyuthesis. This is quite a new and different way from DARWIN and his disciples'. This new theory appears reasonable judging from the recent discovery that amino acid sequences of proteins are under the control of base

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sequence of gene, DNA. Now that it is impossibe to elucidate the detail of the gene structure by itself, the determination of the amino acid sequences of proteins from various kinds of animals is suggestive to deduce the gene structure.

Hemoglobin is a good material for this purpose not merely because it widely distributes among the animal kingdom but because its isolation and purification are relatively easier than those of other proteins. It is known that every hemoglobin from various animals has its own peculiarities on electrophoresis, in alkali-denaturation and so forth, and that these peculiarities are not due to the protoheme of hemoglobin but due to slight differences in structure of the globin part from other hemoglobins. Such differences are presumed to be the accumulation of point mutations caused by the base changes of gene, DNA.

Conerning the primary structure of the globin part, many groups of workers have been studying on various kinds of animals since BRAUNITZER et al.[2] and KONIGSBERG et al.[11] determined that of human hemoglobin. Hemoglobins of primates have been studied by comparing with human hemoglobin. ZUCKERKANDLE et al. presumed that the α-chain of chimpanzee hemoblobin was exactly the same as that of human hemoglobin.[26] They also reported that gorilla hemoglobin differed from human hemoglobin at one point in α-chain and one in β-chain.[27] BUETTNER-JANUSCH et al.[4] analyzed the partial amino acid compositions of tryptic peptides in hemoglobins from hylobates, papio, perodicticus galogo, and lemur, and compared them with that of human hemoglobin, respectively. They reported between human and lemur fulous which is considered to be the evolutionally remotest from human among the primates, there were six amino acid substitutions in α-chain and twenty-three in β-chain of their hemoglobins. The comparative studies of *macaca mulatta* monkey hemoglobin with human hemoglobin show that there are great similarities between them immunologically[20], chromatographically[19], electrophoretically[8], and in alkali-denaturation[5]. However, the slight difference on the fingerprint was proposed by ZUCKERKANCLE et al.[26]

The primary structure of *macaca mulatta* monkey hemoglobin has been investigated in this laboratory.[9],[13],[15],[16],[17],[21],[24] According to the results so far obtained, there are at least eleven differences in the amino acid sequence between human and *macacaa mulatta* monkey hemoglobins. The present author reports here on a partial amino acid sequence of the α-polypeptide chain in *macaca mulatta* monkey hemoglobin.

MATERIALS AND METHODS

1) Preparation of the α-Polypeptide Chain

Hemoglobin was prepared from blood of a *macaca mulatta* monkey

by DRABKIN's method.[6] It was dehemed in acetone containing HCl according to the method of ANSON and MIRSKY.[2] Separation into α- and β-polypeptide chains was performed by countercurrent distribution method with the system of sec-butanol containing 0.08% trichloroacetic acid, propionic acid, and water (8.7: 1.5: 11.0).[18]

2) Preparation of αT9, αT10, and αT11 from the α-Polypeptide Chain

In 8M urea, 1g of the α-polypeptide chain was denatured at 60°C for 45 minutes. After removing urea by dialysis, it was digested with 10mg of trypsin (Worthington Biochemical Corporation, twice-crystallized) at pH 8.0 for four hours at 37°C. The digest was adjusted to pH 6.4 with 1N acetic acid and the insoluble 'core' peptides were precipitated. The soluble peptides in the supernatant were isolated and purified both by column chromatography with Dowex 1×2 as a adsorbent and the acetate buffer containing organic bases such as pyridine, collidine, lutidine, and picoline as a developer, and by paper chromatography with a mixture of n-butanol and acetic acid as a developer.[13] A portion of each tryptic peptide thus obtained was subjected to the amino acid analysis.

3) Digestion of the αT9 with Pepsin

In 30ml of deionized water, 15 μmoles of αT9 was dissolved and denatured at 95°C for five minutes. After cooling, the pH was brought to 2.0 with 0.2N HCl. By the addition of 4mg of pepsin (Sigma Chemical Company, three-time crystallized) which was dissolved in 2ml of 1/16N HCl, this solution was incubated at 37°C for two and a half hours. Then it was adjusted to pH 9.0 with 2.5N NaOH and lyophilized.

4) Isolation of Peptic Peptides from αT9 by Column Chromatography

The resin, Dowex 1×2 (200−400 mesh, Cl-type, Dow Chemical Company) was settled to remove the very fine particles and washed with 1N NH_4OH, water, acetic acid, and water, successively. It was suspended with starting buffer pH 8.5 (1% pyridine, 1% 2.4 lutidine, and 1% α-picoline) and thoroughly evacuated by a vacuum pump. The resin thus prepared was loaded in a column (0.9×60 cm), kept at 37°C, and equilibrated with the above-mentioned buffer overnight.

The sample was dissolved in 20ml of deionized water and the pH brought to 9.0 with 0.1N NaOH. Then it was put on the column. The development was performed at 37°C by using a flow rate of 100 ml per hour. Fraction No. 1 to No.15 were eluted with the starting buffer pH 8.5. The pH-gradient with acetic acid was employed from Fraction No.16. Namely, Fraction No.16 to No.75 were eluted by supplying 0.08 N acetic acid in the reservoir to 600ml of the starting buffer in the mixing chamber. The elution of Fraction No. 76 to the last, the content in the resorvoir was changed into 1.0N acetic acid. All the organic bases used for the developer were purified by distilla-

tion and the deionized water was removed CO_2 by boiling and thoroughly evacuated. The effluent was collected in 8 ml-fractions. The peptides in the effluent was detected by the ninhydrin method according to YEMM and COCKING[25] after an aliquot of each fraction was alkali-hydrolyzed. To. 0.4 ml of each fraction was added 1ml of 2.5N NaOH. This mixture was hydrolyzed at 95°C for two and a half hours, and to this was added 1 ml of 30% acetic acid, 0.5 ml of 0.2M citrate buffer pH 5.0, and 1.2 ml of ninhydrin KCN solution. It was heated at 100°C for 15 minutes and then, after cooling, diluted with 3ml of 60% ethanol. The optical density at 570mμ of each fraction was measured. The effluent at the peaks on the chromatogram was collected respectively, dried under reduced pressure, dissolved again in 3 ml of deionized water, and finally lyophilized.

5) Identification and Purification of Peptides by Paper Chromatography

The descending paper chromatography was performed on a sheet of Toyo filter paper No. 50 (40×40cm) with the upper phase of a mixture of n-butanol, acetic acid, and water (4:1:5). In order to detect peptides the paper was sprayed 0.2% ninhydrin n-butanol solution and heated to color with an iron. PAULI's reaction [23] was also employed for identification of the peptides containing histidine. The Rf Leu value which expresses the relative mobility of a peptide to standard leucine was calculated to show the positions of peptides on the chromatogram.

In case of purification of these peptides, the locations of peptides were first confirmed by guide strips and then the appropriate areas were cut out. Peptides were eluted with 5% acetic acid.

6) Paper Electrophoresis of Peptides

The sample was applied on the center of a sheet of Toyo filter paper No. 51 (10×60cm). The electrophoresis was carried out in an electrophoretic apparatus (Ishidai type) at 2000 V for two hours by using 10% pyridine acetate buffer pH 6.4. Detection and purification of the peptides were performed by the same methods as in the paper chromatography.

7) DNP Method for N-Terminal Amino Acid Analyses of Peptides

A peptide (0.2~0.5μmole) was dissolved in 3ml of 1% $NaHCO_3$ and to this was added 0.05 ml of dinitrofluorobenzene (DNFB). This reaction mixture was incubated at 40°C for two hours. The excess DNFB was extracted with ether and then the aqueous phase acidified with HCl was again extracted with ether. Consequently, dinitrophenol (DNP) which was considered to be a byproduct in the dinitrophenylation was taken off in the ether phase. The DNP-peptide remaining in the aqueous phase was dried, dissolved in constant boiling point HCl, and then hydrolyzed in a sealed tube at 105°C for 20 hours. DNP-amino acids were extracted with 10ml of ether three times from the hydrolysate.

This ether phase was dried under reduced pressure in a heart-shaped flask, the modification of MILLS' apparatus[10]. Subsequently, this flask was connected to the cold finger and evacuated by a vacuum pump. Identification of the DNP-amino acids were carried out by two-dimensional paper chromatography. Namely, the sample dissolved in a small portion of acetone was applied on a sheet of Toyo filter paper No. 51 (40×40cm). Development was performed by ascending method; the first dimension with the upper phase of a mixture of n-butanol and 1N NH_4OH (1:1); the second, with 0.5M phosphate buffer.

8) PTC Method for Amino Acid Sequence Analyses of Peptides

The phenylisothiocyanation of peptides was performed in 66% pyridine according to the modification of EDMAN's original method.[7] Formation of PTH-amino acids were carried out with trifluoroacetic acid. The PTH-amino acids were not identified, however, the amino acid compositions of the remaining peptides were analyzed.

A peptide (0.5−3.0μmole) was dissolved in 2.5ml of 66% pyridine and poured in a heart-shaped flask. To this was added phenylisothiocyanate (PTC) and the reaction mixture allowed to stand at 37°C for three hours. It was dried under reduced pressure. The flask was connected to the cold-finger and incubated by a vacuum pump to remove the excess PTC. To this was added 1ml of trifluoroacetic acid. This was allowed to stand at room temperature for three and a half hours. Trifluoroacetic acid was removed under reduced pressure and the residue was dissolved in 3ml of deionized water. From this solution, PTH-amino acids were extracted three times with 3ml of benzene. A portion of the peptide in aqueous phase was hydrolyzed with HCl, and the amino acid composition was analyzed. The remainder was dried under reduced pressure and used in the next step of the PTC method. Long peptides had to be further purified by paper electrophoresis.

9) Amino Acid Analyses of Peptides

A peptide was dissolved in 4ml of constant boiling point HCl and hydrolyzed at 105°C for 22−24 hours in a sealed tube. The hydrhlysate was dried and removoed HCl thoroughly. Its amino acid composition was examined on an amino acid analyzer, Hitachi KLA 2 with a 7.5cm column and a 50cm column. The analysis values were shown in molar ratios without correction for losses during the procedure.

RESULTS AND DISCUSSION

Table I shows the amino acid compositions of the tryptic peptides, αT9, αT10, αT11, and their yields obtained from the tryptic hydrolysate of 1g of the α-polypeptide chain of *macaca mulatta* monkey hemoglobin. It was previously reported that αT9 had four residues of aspartic acid and one residue of glutamic acid,[13] however, in this experiment the analysis values of aspartic acid and glutamic acid in αT9 were 4.71

Table I
Amino acid compositions of αT9, αT 10, and αT11, and their yields from 1000 mg of the α-polypeptide chain.

	α T9	α T10	α T11
Lys	1.18		0.97
His	2.87	0.95	
Arg			2.14
Asp	4.71		
Thr	0.88		
Ser	1.81		
Glu	0.21		
Pro	1.11		0.99
Gly	1.18		
Ala	6.08		
Val	2.77		1.95
Met	0.53		
Leu	5.19	1.05	
Tyr			
Phe			0.95
Try			
Yield	18μmoles	32μmoles	19μmoles

and 0.21, respectively. In the process of sequence determination, no glutamic acid residue was recognized in αT9.

1) Amino Acid Sequence of αT9

The N-terminal residue was known to be valine by DNP method. Fragmentary peptides were obtained from the peptic hydrolysate of αT9. They were fractionated into eight peaks as shown in Fig. 1 by column chromatography. Each peptide in a peak was purified by paper chromatography. Ten main peptides were obtained as shown in Fig. 2. Table II gives the amino acid compositions of these peptides. The sequence determination was carried out through the following procedure. The yields through the procedure

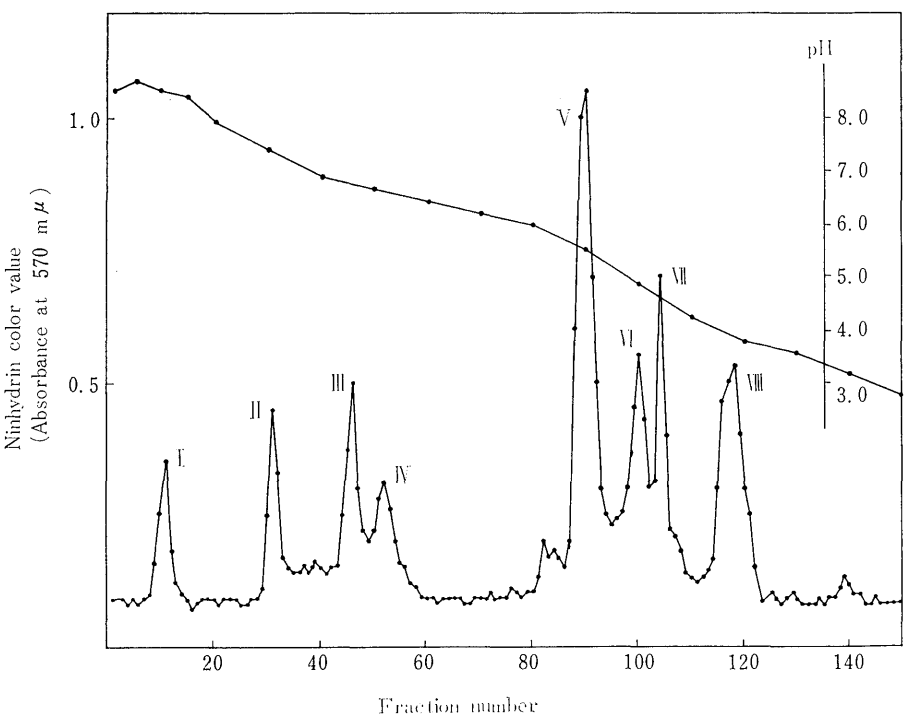

Fig. 1 Column chromatography of the peptic peptides from αT9

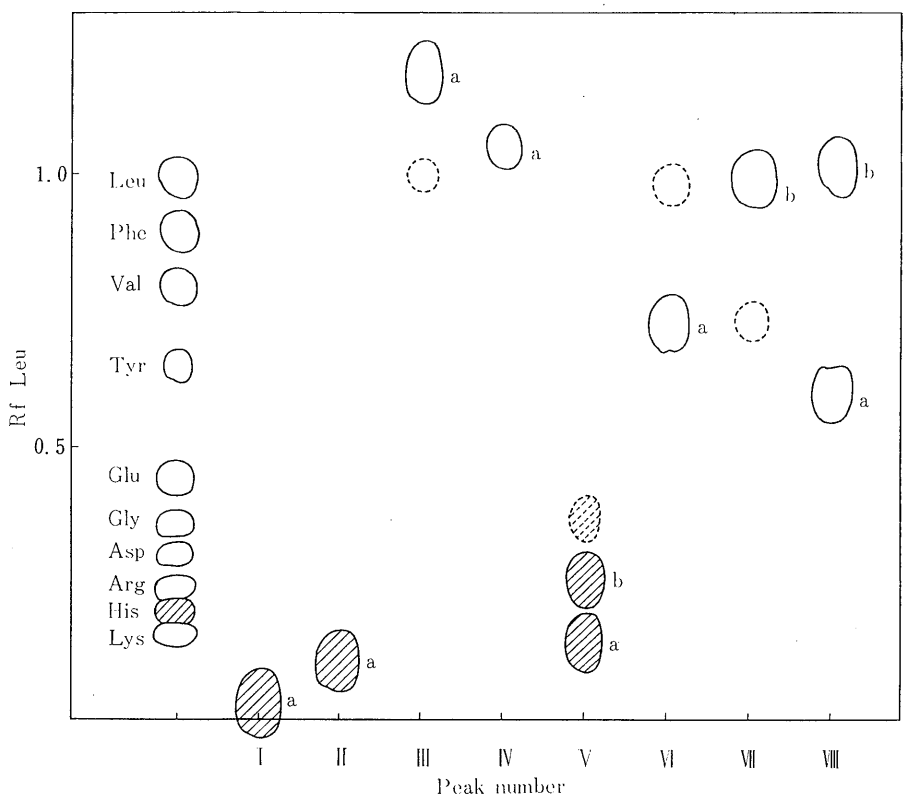

Fig.2 Paper chromatography of the peptic peptides from αT9.

Table II Amino acid compositions of the peptic peptides from α T9

	P-Ia	P-IIa	P-IIIa	P-IVa	P-Va	P-Vb	P-VIa	P-VIIb	P-VIIIa	P-VIIIb
Lys	1.07	1.08								
His	1.89	1.91			1.11	1.02				
$MetO_2$					+	+				
Asp		1.01			2.59	2.59	0.96	1.00	1.03	1.03
Thr			0.97					0.97		
Ser		0.82		0.92	0.71	0.70			0.90	0.94
Glu					0.19	0.21				
Pro					1.14	1.08				
Gly					1.20	1.10				
Ala	1.03	1.19		1.00	2.71	2.69	2.09	2.27		
Val		1.01			2.01	2.17	0.94	0.86		
Leu			2.03	1.08	1.15	2.19		1.03	1.07	2.02

from peptic digestion to amino acid analysis are given in percentage.

(a) αT9 P-Ia Rf Leu 0.04 29%
Two steps of PTC method were performed.

	Lys	His	Ala
Step 1	1.08	1.19	0.92
Step 2	1.00	1.13	0.21

Since lysine is supposed to be the C-terminal residue of αT9, this peptide has a sequence of His-Ala-His-Lys and is located at the C-terminus of αT9.

(b) αT9 P-IIa Rf Leu 0.10 12%

It is supposed from the composition in Table II that this peptide is a combination of P-Ia and P-VIIIa.

(c) αT9 P-IIIa Rf Leu 1.18 10%

Two steps of PTC method gave the following results.

	Thr	Leu
Step 1	1.00	1.27
Step 2	0.18	1.00

Accordingly, the sequence was determined to be Leu-Thr-Leu.

(d) αT9 P-IVa Rf Leu 1.04 10%

Two steps of PTC method were carried out.

	Ser	Ala	Leu
Step 1	0.09	0.99	1.01
Step 2	0.00	0.19	1.00

The sequence was, therefore, Ser-Ala-Leu.

(e) αT9 P-Va Rf Leu 0.15 23%
αT9 P-Vb Rf Leu 0.25 27%

These two peptides consist of 13 and 14 amino acid residues, respectively as shown in Table II and resemble each other except that P-Va has leucine one residue less than P-Vb. The remaining peptide of P-Vb after the first step of PTC method had the same composition as that of P-Va. In the previous experiment, αT9 was digested with pepsin for three hours and a peptide resembling this P-Va was obtained. The amino acid composition was His: 0.93, Met: (+), Asp: 2.72,Ser: 0.95, Pro: 1.20, Gly: 1.19, Ala: 2.12, Val: 1.98, and Leu: 1.08. The sequence analysis was tried by digesting this peptide with pronase P for 16 hours but failed only obtaining some dipeptides and amino acids. This peptide was also digested with papain for 10 hours to obtain two kinds of peptides whose compositions were a) Gly: 0.86, Ala: 1.13, Val: 1.01, and b) His: (+), Met: (+), Asp: 2.41, Pro: 1.18, Ala: 1.16, Val: 1.21.

In this experiment, the amino acid sequence of this part was determined by the employment of PTC method on P-Va and P-Vb. Table III gives the results obtained by PTC method together with the amino acid composition of P-Vb. The peptide after the 5th and 8th step of PTC method had to be purified by paper electrophoresis. The electric charge of the peptide on each step was examined by electrophoresis to distinguish between aspartic acid and asparagine. As the result, the eleventh residue from the N-terminus of this peptide was known to be

Table III Results of PTC method on αT9 P-Vb

	His	$MetO_2$	Asp	Ser	Glu	Pro	Gly	Ala	Val	Leu
P-Vb	1.02	(+)	2.59	0.70	0.21	1.08	1.10	2.69	2.17	2.19
Step 1	0.98	(+)	2.33	1.03	trace	1.02	1.22	2.78	1.98	1.21
Step 2	1.02	(+)	2.49	0.78	0.00	0.85	1.18	2.11	1.79	1.06
Step 3	0.93	(+)	2.62	0.84	0.00	0.89	1.11	1.97	1.06	1.13
Step 4	0.97	(+)	2.96	0.85	0.00	0.93	0.58	2.07	1.05	1.16
Step 5	0.51	(+)	2.83	0.87	0.00	0.98	0.08	2.02	1.01	0.99
Step 6	0.00	(+)	2.98	0.86	0.00	1.14	0.09	2.04	0.13	1.07
Step 7	not measured	(+)	2.23	0.98	0.00	1.00	0.00	2.02	0.00	1.00
Step 8	〃	(+)	1.63	1.04	0.00	1.03	0.00	1.91	0.21	1.02
Step 9	〃	(−)	1.12	0.88	0.00	0.95	0.00	2.13	0.00	0.92
Step 10	〃	(−)	1.22	0.91	0.00	0.00	0.00	1.90	0.00	1.13
Step 11	〃	(−)	0.43	1.00	0.00	0.00	0.00	1.81	0.00	1.19
Step 12	〃	(−)	0.21	0.89	0.00	0.00	0.00	1.21	0.00	1.00
Step 13	〃	(−)	0.25	0.91	0.00	0.00	0.00	1.09	0.00	0.53

asparagine. Therefore, the sequence was as follows; Leu-Ala-Val-Gly-His-Val-Asp-Asp-Met-Pro-Asp (NH_2)-Ala-Leu-(Ser, Ala). As for the order of serine and alanine at the C-terminus, the sequence, Ser-Ala, was probable from the result on αT9 P-IVa.

(f) αT9 P-VIa Rf Leu 0.72 25%

Three steps of PTC method were performed.

	Asp	Ala	Val
Step 1	1.08	1.92	0.12
Step 2	0.93	1.22	0.00
Step 3	0.41	1.00	0.00

The elution behavior on the column chromatogram and the electrophoretic behavior on the paper electrophoregram showed that this peptide was acidic. The sequence was therefore determined to be Val-Ala-Asp-Ala.

As described previously the N-terminal residue of αT9 had known to be valine and only this peptide contained it. Therefore, it was considered that this peptide was located at the N-terminus of αT9.

(g) αT9 P-VIIb Rf Leu 1.00 19%

The sequence of this peptide was deduced to be Val-Ala-Asp-Ala-Leu-Thr judging from the composition in Table III and the sequences of P-VIIIb and P-IIIa.

(h) αT9 P-VIIIa Rf Leu 0.60 5%

Two steps of PTC method were carried out.

	Asp	Ser	Leu
Step 1	0.96	0.22	1.04
Step 2	0.24	0.00	1.00

Since this peptide was known to be acidic, the sequence was Ser-

Asp-Leu.

(i) αT9 P-VIIIb Rf Leu 0.98 21%

Three steps of PTC method were carried out.

	Asp	Ser	Leu
Step 1	0.98	0.92	1.10
Step 2	0.95	0.10	1.05
Step 3	0.20	0.00	1.00

This result indicates that this peptide was the combination of a leucine residue and P-VIIIa at its N-terminus. The sequence was Leu-Ser-Asp-Leu.

The amino acid sequences of the ten peptides obtained by peptic digestion of αT9 were determined as described above. The peptide sequence in αT9 was determined as shown Fig. 3, considering that P-VIa and P-VIIb had known to be located in the N-terminus of αT9 and P-Ia and P-IIa, in the C-terminus.

The comparion of the amino acid sequence of this αT9 with that of human hemoglobin showed two differences between them. Namely, the seventh residue from the N-terminus of αT9 is asparagine in human hemoglobin, but leucine in *macaca mulatta* monkey hemoglobin. Similarly the tenth from the N-terminus is alanine in the former but glycine in the latter. It was elucidated that αT9 of *macata mulatta* monkey hemoglobin had no glutamic acid residues.

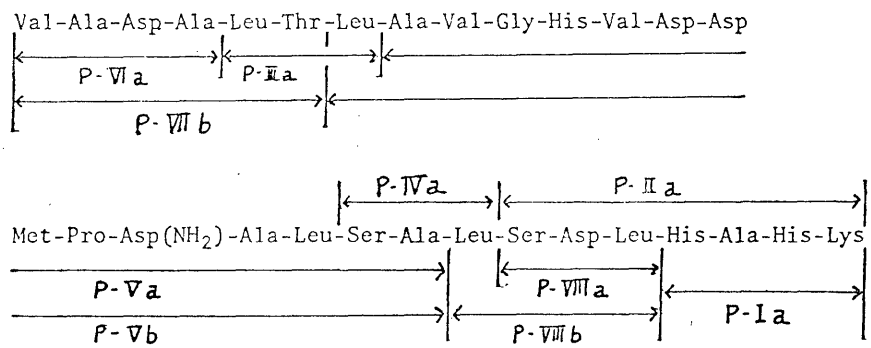

Fig. 3 The amino acid sequence of αT9

2) Amino Acid Sequence of αT10

As shown in Table I, this is a dipeptide containing leucine and arginine. Since it was a tryptic peptide, the sequence was deduced to be Leu-Arg.

It was confirmed by DNP method for the N-terminal analysis.

3) Amin Acid Sequence of αT11

This peptide containing seven amino acids as shown in Table I was subjected to five steps of PTC method for the sequence determination.

	Asp	Pro	Val	Phe	Lys
Step 1	2.06	1.00	0.97	0.98	not determined
Step 2	1.18	0.94	0.95	0.92	〃
Step 3	1.12	0.19	0.92	0.95	〃
Step 4	1.09	0.00	0.28	0.91	〃
Step 5	0.48	0.00	0.14	1.00	〃

This peptide was originally neutral, however, it was known by paper electrophoresis that after the second step of PTC method the remaining peptide after each step changed into basic. This indicates that aspartic acid was eliminated in the second step and, asparagine, in the fifth step. Since lysine was supposed to be the C-terminal residue, the sequence of αT11 was determined to be Val-Asp-Pro-Val-Asp (NH_2)-Phe-Lys. This is quite similar to the corresponding peptide in human hemoglobin.

CONCLUSION

The sequence of thirty-eight amino acid residues contained in the tryptic peptides, αT9, αT10, αT11 of the α-polypeptide chain in *macaca mulatta* monkey hemoglobin was determined. The comparison of the corresponding sequences between human and *macaca mulata* monkey hemoglobins demonstrated two amino acid substitutions in αT9. They were Asp (NH_2) [human] ⟶ Leu [monkey] and Ala [human] ⟶ Gly [monkey]. The sequences of other 36 residues were all the same as each other.

ACKNOWLEDGEMENT

The author wishes to express his cordial gratitude to Prof. Dr. G. MATSUDA and Dr. T. MAITA who gave him constant and useful advice during this work, and also to Miss S. HAZAMA for amino acid analysis and to Miss S. ARAKAWA for her help in preparing this paper.

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